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Pathogens and autoimmune hepatitis

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Summary

Autoimmune hepatitis (AIH) is a severe form of hepatitis resulting in the autoimmune-mediated destruction of the liver parenchyma. Whereas many of the immunopathogenic events have been elucidated and some of the drivers of the disease have been identified, little is known about the aetiology of the disease. There are certain risk factors, such as particular human leucocyte antigen (HLA) haplotypes, that enhance the susceptibility for AIH or influence the severity of the disease. However, as for many other autoimmune diseases, the mere presence of such risk factors does not warrant the occurrence of the disease. Not all individuals carrying risk factors develop AIH, and not all patients with AIH are carriers of high-risk alleles. Thus, additional environmental factors need to be considered as triggers for AIH. Environmental factors include diet, sunlight exposure, stress, medication and hygiene, as well as pathogen infections and vaccinations. This review discusses if pathogens should be considered as triggers for the initiation and/or propagation of AIH.

Keywords: autoimmunity, CYP2D6, Inflammation, molecular mimicry, virus infection

Autoimmune hepatitis (AIH)

AIH is a serious inflammatory liver disease that results in the autoimmune-mediated destruction of the liver parenchyma, chronic inflammation and fibrosis [1–4]. According to the World Health Organization, AIH has an annual incidence of approximately two in 100 000 individuals and a prevalence of 15 cases per 100 000 people worldwide (https://www.who.int/ipcs/publications/ehc/ehc236.pdf). Like many other autoimmune diseases, AIH is more frequent in women (female : male ratio, 3·6 : 1), occurs in children and adults of all ages and is not restricted to certain ethnic groups [5]. The main features of AIH is an interface hepatitis, presence of autoantibodies against liver autoantigens and elevated immunoglobulin (Ig)G and aminotransferase levels. However, the clinical spectrum is variable, and ranges from asymptomatic presentations to severe hepatitis with features similar to acute viral hepatitis or fulminant hepatic failure [1–4,6,7]. In addition, the existence of so-called overlap syndromes of AIH with other autoimmune liver diseases, such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), make a conclusive diagnosis of AIH somewhat difficult [8]. The International AIH Group (IAIHG) agreed on a diagnostic scoring system to aid in the diagnosis of AIH [9]. However, in clinical practice the scoring system, which comprised 13 different parameters and a total of 30 scoring elements, proved too complex. Therefore, in 2008 the IAIHG produced a simplified scoring system that consists of only four parameters, including increased immunoglobulin G (Ig) concentration (hypergammaglobulinaemia), absence of viral markers, typical histological features compatible with AIH and presence of specific autoantibodies [10,11]. Serum aminotransferases are frequently elevated in AIH and in clinical routine are the first indicators of liver disease. However, as aminotransferases are markers of hepatocyte destruction that are found to be elevated in many different forms of liver disease, they have

therefore not been considered in the simplified scoring system.

One of the main diagnostic criteria of AIH and its subtypes is the presence of specific autoantibodies to liver autoantigens [12,13]. In general, the presence of anti-nuclear (ANA) and/or anti-smooth muscle (SMA) autoantibodies characterizes AIH type 1 (AIH-1), whereas type 1 liver/kidney microsomal autoantibodies (LKM-1) are the hallmark of AIH type 2 (AIH-2) [1–4]. Patients with AIH-1 and AIH-2 differ in clinical presentation and course of disease [14]. However, a recent study with 78 AIH-patients demonstrated that, with the exception of differences in the presence of individual autoantibodies, adult patients diagnosed with AIH-1 or AIH-2 may also share a similar clinical phenotype [15]. Furthermore, patients carrying autoantibodies directed against soluble liver antigen (SLA) and liver and pancreas antigen (LP) (anti-SLA/LP antibodies) have been classified historically as type 3 AIH (AIH-3) patients. In the meantime, subtyping into AIH-3 is obsolete, as anti-SLA/LP antibodies are often present in context with other autoantibodies pointing towards AIH-1 and the course of disease is similar to AIH-1 [16].

There are many recent reviews that focus on autoantibodies in patients with AIH [12,13,17], therefore we will simply highlight briefly the most important aspects. Although often used to classify patients with AIH-1, ANA are not specific for AIH, but are also present in patients with PBC, systemic sclerosis (SSc), drug-induced hepatitis, chronic hepatitis B or C and non-alcoholic fatty liver disease (NAFLD) [17]. However, the term ANA is more of a descriptive term that simply states a reactivity of antibodies to any structure present in the nucleus and, indeed, the diffuse groups of ANA consist of several different antibodies recognizing DNA, centromers, histones, small nuclear ribonucleoproteins (sn-RNPs) and cyclin A [18,19]. The mere presence of ANA may be compatible with, but not a *bona fide* diagnostic marker for, AIH-1 [17]. Similarly, SMA that are reactive to filamentous actin (F-actin) only represent a reliable diagnostic tool for AIH-1 if the staining pattern is evaluated carefully. Although SMA have been found in the sera of patients with other liver diseases with an autoimmune or viral background, the titres are usually higher in AIH-1 [12,17].

The hallmark for AIH-2 is the presence of anti-LKM-1 antibodies that react to three proteins of the microsomal compartment, the 2D6 isoform of the cytochrome P450 enzyme family (CYP2D6) [20,21], ERp57 and carboxylesterase 1 (CES1) [22]. Reactivity to CYP2D6 has been identified first, and the presence of anti-LKM-1 antibodies is considered diagnostic for AIH-2 in hepatitis C virus (HCV)-negative patients. Reactivity to CYP2D6 has also been found in patients with chronic hepatitis C [23–25]. However, as will be discussed below, HCV infection might also play a role in the aetiology of AIH. Additional autoantibodies include peripheral antinuclear neutrophil antibodies (pANNA) [also termed 'atypical' peripheral anti-neutrophil cytoplasmic antibodies (pANCA)], anti-liver and pancreas antigen (LP) antibodies, liver cytosol type 1 antibodies (LC-1), type 2 or type 3 liver/kidney microsomal antibodies (LKM-2 and LKM-3, respectively), anti-liver-specific membrane lipoprotein (LSP) antibodies and anti-liver membrane antibodies (LMA) [26–32]. Similar to anti-LKM-1 antibodies, anti-LC1 antibodies have been characterized extensively [33] at its major target identified as the formiminotransferase cyclodeaminase (FTCD) [34]. In addition, the major autoantigen recognized by anti-SLA/LP antibodies has been identified as the UGA serine tRNA–protein complex (tRNP(Ser)Sec) [28,35]. The text has been modified accordingly and the refs have been added. As well as the presence of autoantibodies, a histological evaluation of liver biopsies is a prerequisite for a reliable diagnosis of AIH. The histological hallmark of AIH is an interface hepatitis with piecemeal necrosis affecting patches of hepatocytes. Often, such regions are characterized by plasmacytosis (infiltrating plasma cells), hepatocyte rosetting and emperipolesis [1–4].

The European Association for the Study of the Liver (EASL) recommends a glucocorticoid treatment with prednisone or prednisolone alone or in combination with azathioprine as the standard therapy of AIH [2]. For non-responders to the standard therapy, the next-generation glucocorticoid budesonide might represent an alternative. However, budesonide administration should be considered with care, as the lack of efficient first-pass hepatic clearing of budesonide might result in undesired side effects in patients with cirrhosis or peri-hepatic shunting [2]. It has been reported that a combination therapy with budesonide and azathioprine resulted in fewer side effects than the conventional prednisone/azathioprine therapy in AIH patients without cirrhosis [36]. Further alternative immunosuppressive regimens include the calcineurin inhibitors cyclosporin A and tacrolimus [37,38] as well as the cytostatic immunosuppressant drug mycophenolate mofetil (MMF), which has been demonstrated to be safe and effective as first-line or rescue therapy [39]. However, the use of MMF is predominantly recommended as a second-line therapy in cases of azathioprine intolerance [2]. As for most autoimmune diseases, the treatment of AIH might be required for decades and a short-term standard therapy is not very effective. AIH resolution is rarely achieved in less than 12 months and withdrawal of therapy after only 2 years of treatment results in relapses in 85% of cases [5]. Thus, long-term standard therapy

carries the risk of significant corticosteroid-specific and azathioprine-related side effects.

Genetic predisposition

As to be expected, the human leucocyte antigen (HLA) haplotype is the dominant factor influencing the risk to develop AIH. It has long been recognized that both variants of AIH are associated with major histocompatibility complex (MHC) class I HLA-B8 and with MHC class II HLA-DR3 (*DRB1*03:01*). In addition, AIH type 1 is also associated with HLA-DR4 (*DRB1*04:01*), whereas AIH type 2 is associated with HLA-DR7 (*DRB1*07:01*) and HLA-DQ2 (*DQB1*02:01*) [40–46]. However, distinctive susceptibility variants have been reported for different ethnic groups [46]. A genomewide association study (GWAS) on Dutch and German patients confirmed *DRB1*03:01* and *DRB1*04:01* as the primary and secondary susceptibility loci for AIH-1 [47]. Although a number of GWAS identified several additional risk factors for PBC and PSC, only one additional risk factor has been detected for AIH; namely, the *SH2B3* locus encoding Lnk, an adaptor protein involved in multiple cell surface signalling pathways [47,48]. Mutations in *SH2B3* are implicated in myeloproliferative disorders including malignancies [49]. However, besides the GWAS data, several other associations have been suggested previously. Of particular interest are the reported associations of AIH with cytotoxic T lymphocyte antigen 4 (CTLA-4) [50], vitamin D receptor (VDR) [51], Fas [52], and tumour necrosis factor (TNF)-α [53]. However, these associations are yet to be confirmed, and they did not appear in the above-mentioned GWAS of de Boer *et al.* [47].

Interestingly, the presence of certain HLA alleles also has an influence on the course of AIH: patients carrying the HLA-B8 allele develop a more severe inflammation with higher levels of AST and bilirubin, more commonly had histological features of bridging necrosis, multi-lobular necrosis and cirrhosis and are more likely to have a relapse after treatment [54]. The presence of HLA-DR3 is associated with a lower remission rate, a higher relapse frequency and a frequent requirement for liver transplantation [55]. Further, patients carrying *DRB1*03:01* develop a more pronounced hypergammaglobulinaemia [56] and are associated with a significantly higher risk for adverse treatment outcome with subsequent requirement for liver transplantation or resulting in death from acute liver failure [42,57]. In contrast, individuals with HLA-DR4 are associated with a more favourable clinical outcome characterized by a higher rate of complete remission and a lower frequency of cirrhosis [58].

Pathogens and autoimmune disease

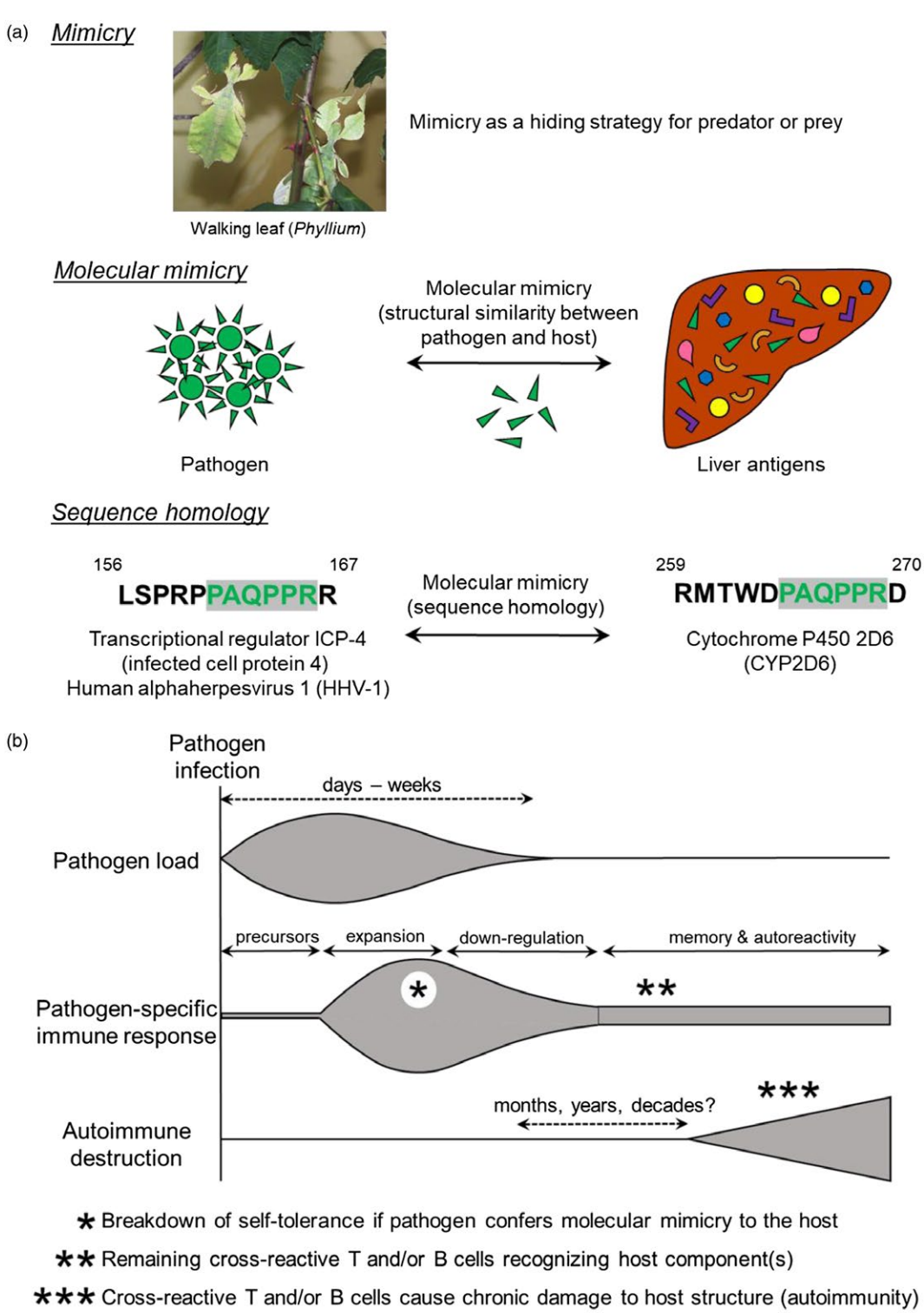
Pathogens have been implicated in the aetiology of many autoimmune diseases, as pathogen infections usually result in the initiation of an innate as well as an adaptive immune response. Depending on the nature of the pathogen, the ensuing immune response has more of a type 1 phenotype (aggressive, cytotoxic), as required for the elimination of intracellular pathogens such as viruses, or elicits more of a type 2 phenotype (allergic), as required for the elimination of extracellular parasites such as helminths. Thus, viruses are the prime suspects for the initiation and/or propagation of an autoimmune process, which for most diseases is characterized by an aggressive type 1 immune response. Whereas, for virtually all autoimmune diseases, associations with a broad variety of pathogens have been reported, firm proof that such disease-associated pathogen infections indeed play a role in the initiation and/or propagation of the autoimmune destructive processes is often missing. There are several circumstances that make it difficult to find a definitive proof. First, not all patients have a documented history for a particular pathogen infection, and not all individuals encountering such a pathogen are diagnosed with the associated autoimmune disease. Secondly, some patients might indeed have encountered a particular pathogen in the past, but at the time of disease diagnosis no traces of a former pathogen infection can be found, due to a total elimination of the pathogen and the absence of a significant antibody titre. Thus, such non-chronic pathogen infections are considered as 'hit-and-run' events. Thirdly, another way by which a bystander inflammation might be induced is the breakdown of barriers preventing commensal bacteria to escape the gut. A recent report suggested that certain gut pathobionts drive autoimmune processes in peripheral organs [59]. In this study the translocation of the gut pathobiont *Enterococcus gallinarum* to the liver triggered autoimmunity in transgenic mice prone to develop systemic lupus erythematosus (SLE) in a bystander fashion by inducing cytokines, autoantigens, endogenous retrovirus proteins and other autoimmune-promoting factors [59]. Fourthly, infections by more than one pathogen might be required to precipitate an autoimmune disease. Thus, some pathogens might initiate autoimmune processes that fall short of resulting in clinical disease, whereas other pathogens might only accelerate, rather than initiate an autodestructive process. Fifthly, trophism and pathogen strain are other important factors. Although an infection of the target organ might not be an absolute prerequisite for the development of an organ-specific autoimmune disease, the ensuing local inflammation might be required for a subsequent autoimmune disease. Finally,

certain pathogen infections, especially by helminths, might protect from adverse immune reactions, rather than initiate or enhance them [60]. Thus, the entire history of pathogen infections might determine the overall immune status that may or may not result in the development of an autoimmune disease [61]. In addition to directly influencing the immune response to autoantigens in autoimmune diseases, host factors such as the HLA haplotype, as well as the microbiome, might influence the pathogen-specific immune response and therefore affect mechanisms responsible for pathogen-induced self-tolerance breakdown.

Several mechanisms have been suggested for how pathogens might initiate and/or accelerate autoimmune processes. First, infections with intracellular pathogens, such as viruses, often cause direct damage to the infected cells and trigger an up-regulation of MHC molecules at the surface of these infected cells, as well as on professional antigen-presenting cells (APC) that act as scavengers of the damaged infected cells. Up-regulated MHC expression comes along with enhanced antigen presentation of peptides derived from both the infecting pathogens as well as the host cells. Thereby, previously sequestered host epitopes might be presented in a way to activate self-reactive T cells. Secondly, pathogen infections cause an activation of the host defence mechanisms that aims at a rapid elimination of the invading foreign organism. The resulting acute and/or chronic inflammation includes an enhanced release of prostaglandins, cytokines and chemokines as well as an activation of the innate and adaptive immune system. Thereby, only the adaptive immune response is pathogen-specific, whereas other inflammatory factors might cause bystander effects that provide a 'fertile field' for autoimmunity to develop [62]. Thirdly, T cells might become activated by superantigens that cross-link MHC and T cell receptor (TCR) molecules independently of a specific antigen epitope recognition [63]. Some of such polyclonally activated T cells might react to self-components. Fourthly, infection by pathogens sharing a structural similarity to host structures might elicit a pathogen-specific immune response that cross-reacts to host components. Such a molecular mimicry between pathogen and host provides the basis for a hypothesis of how pathogens that have been associated with a human autoimmune disease might be involved in the breakdown of self-tolerance [64,65].

Molecular mimicry

In the early 1960s, Rowley and Jenkin observed an antigenic cross-reactivity between parasite and host, and suggested a concept of protective molecular mimicry by which the pathogen would be tolerated because of its similarity to the host [66]. This concept of molecular mimicry has been transferred to the field of autoimmunity by Damian, who suggested that such a similarity between pathogen and host might result in autoimmunity if the 'hiding strategy' of the pathogen fails. Thereby, the pathogen is not being tolerated and the ensuing aggressive immune response directed against the pathogen also attacks similar target structures of the host [67]. Figure 1 shows the concept of molecular mimicry as a possible mechanism of how pathogens might be involved in the aetiology of autoimmune diseases. Later, it was found by Oldstone and colleagues that molecular mimicry is a widespread phenomenon. They screened more than 600 monoclonal antibodies that have been raised against 11 different viruses, and found that 3·5% reacted to specific target structures in uninfected mice [68]. Since then, many cases of molecular mimicry have been postulated. Often, sequence homologies have been reported between a specific host autoantigen and a pathogen that has been associated with the corresponding human autoimmune disease or the presence of cross-reactive antibodies and/or T cells [64,65]. However, there are only very few cases where there is sufficient proof suggesting that an observed structural similarity between pathogen and host indeed plays a role in the aetiology of the disease. In Guillain–Barré syndrome (GBS), an association has been found with an infection with *Campylobacter jejuni* that shares structural homology of its lipooligosaccharide with the peripheral nerve GM1 ganglioside. Patients with GBS indeed carry autoantibodies (cross-) reactive to both lipooligosaccharide as well as the GM1 ganglioside. Importantly, immunization of rabbits with either purified gangliosides or *C. jejuni* lipo-oligosaccharide triggers GBS-like disease [69]. A second example is the molecular mimicry between the M protein of *Streptococcus pyogenes* and human cardiac myosin. Patients with rheumatic heart disease-related valvulitis generate antibodies and T cells that react to both the streptococcal M protein and cardiac myosin, and rats immunized with streptococcal M protein also develop valvulitis [70]. With regard to autoimmune liver diseases, it has been found that molecular mimicry might play a role in the aetiology of PBC, as anti-mitochondrial antibodies (AMA) of PBC patients that are directed mainly against the lipoic acid moiety of the 2-oxoacid dehydrogenase enzyme family are cross-reactive with components of the bacterium *Novosphingobium aromaticivorans*, as well as the cosmetic food additive 2-octoynoic acid (2-OA). Indeed, when 2-OA is coupled to bovine serum albumin as carrier and injected into C57BL/6 mice, a PBC-like disease characterized by autoimmune cholangitis, anti-mitochondrial antibodies and infiltration of the liver by activated CD8 T cells is induced [71].



* Breakdown of self-tolerance if pathogen confers molecular mimicry to the host

** Remaining cross-reactive T and/or B cells recognizing host component(s)

*** Cross-reactive T and/or B cells cause chronic damage to host structure (autoimmunity)

Fig. 1. Molecular mimicry. (a) Mimicry describes a hiding strategy of certain animals that adapt features of the surrounding environment in order not to be seen by its predator or prey. Similarly, molecular mimicry describes the structural similarity between two molecules. Initially suggested as a hiding strategy of parasites to avoid being attacked by the host immune system, molecular mimicry is also connected to the breakdown of self-tolerance upon infection with a pathogen that shares a structural similarity to a self-antigen. Such a similarity can be conformational at the surface of certain antigens, including the three-dimensional-structures of proteins, lipids, DNA or prosthetic groups, as well as linear/sequential as occurring predominantly in consecutive amino acid sequences of proteins. An example for molecular mimicry on the level of consecutive amino acid sequences is the structural similarity between ICP-4 protein sequence aa156–167 of HHV-1 and the human CYP2D6 protein sequence aa 259–270, that both contain a core sequence of six identical amino acids. (b) The breakdown of self-tolerance due to an infection with a pathogen that confers molecular mimicry to (a) host autoantigen(s) is thought to include the following steps. A pathogen infection elicits an immune response with the goal to eliminate the invading organism. The pathogen might be eliminated within a few days or weeks or alternatively might persist for an extended period. In response to the pathogen infection, specific precursors will expand and increase the magnitude of the immune response to eliminate the pathogen, followed by down-regulation to avoid chronic inflammation. In the case of chronic infection, the immune response is insufficient for a total elimination resulting in a balance between the remaining pathogen load and the insufficient immune response that is too weak to eliminate the pathogen, but is at least sufficient to prevent the pathogen-induced death of the host. Due to the structural similarity between pathogen and host, cross-reactive B cells/antibodies and/or T cells that were generated initially to eliminate the pathogen would also begin attacking similar structures of the host, resulting in autoimmune disease during a period of months, years or even decades after pathogen infection.

Pathogens and autoimmune hepatitis

As mentioned above, there is a huge difference between an observed association of pathogen infections and the occurrence of autoimmune disease and an actual proof for pathogens to be involved in the pathogenesis of a disease. However, due to the described difficulties in finding hard evidence, such associations are often the only indication for an influence of environmental triggering factors such as pathogens.

Hepatitis viruses

Naturally, hepatitis viruses are the first suspects that come to mind when considering pathogens as triggers for AIH. Liver-trophic pathogens such as hepatitis viruses cause direct damage to the liver and result in local inflammation. Therefore, it does not come as a surprise that hepatitis A, B and C viruses have been associated with AIH. In the early 1990s an association between hepatitis A virus (HAV) infection and AIH has been demonstrated in a cohort of 58 first-degree relatives of AIH patients. Three relatives displayed subclinical hepatitis A and, interestingly, two of these three patients subsequently developed AIH within 5 months [72]. Although the two patients were negative for the most frequent autoantibodies ANA or LKM-1 they generated anti-LSP antibodies, which are present in up to 88% of AIH patients [26] and are commonly found to be transiently present in patients with acute hepatitis A [73]. The most frequent association between pathogen and AIH is documented for HCV. Typical autoantibodies that are found in patients with AIH-1 have also been found in chronic hepatitis C patients. In particular, SMA and ANA have been found in up to 66 and 41% of HCV patients, respectively [74]. In addition, both SMA as well as ANA have been detected in the sera of some patients infected with HBV [74,75] or hepatitis D virus (HDV) [76]. It is important to note that in contrast to well-defined autoantibodies such as the LKM-1, which recognize CYP2D6, SMA and ANA describe a heterologous antibody reactivity to smooth muscle actin and nuclear antigens, respectively. Thus, a detailed evaluation of the histological patterns of SMA revealed that AIH-1 SMA react to arterial vessels as well as renal glomeruli and tubules, whereas SMA from HCV-infected patients tend to stain only the arterial vessels [76]. Further, the nuclear staining of ANA appears homogeneous for ANA from patients with AIH-1 and speckled with hepatitis C patients' ANA [76]. These findings indicate that the presence of SMA and ANA in patients infected with hepatitis viruses does not provide any evidence for those viruses to be involved in the aetiology of AIH. This is particularly true for ANA, which comprise reactivities to many different nuclear antigens, including nuclear body-associated protein sp100 or the nuclear pore membrane protein gp120 (both present in the majority of PBC patients), DNA, centromers, histones, sn-RNPs and cyclin A and are, as well as being present in patients with AIH, PBC and chronic hepatitis B or C infection, are also found in drug-induced hepatitis and in patients with non-alcoholic fatty liver disease (NAFLD) [17]. Thus, a different recognition pattern could most probably be simply the result of a distinct specificity for autoantigens that have a different subnuclear distribution. Molecular mimicry of amino acid sequences has been reported between regions of the HCV polyprotein and regions of three smooth muscle proteins (vimentin, smoothelin, myosin) and two nuclear antigens (matrin, histon H2A) [77]. A majority of sera from patients infected with HCV that also generated SMA or ANA reacted to some of the above-mentioned mimicking host antigens [77]. This study demonstrates that HCV infection may indeed result in

the generation of cross-reactive antibodies, but is not proof that they also cause AIH, especially as none of the patients developed the disease.

A large study from Brazil demonstrated the presence of intense interface hepatitis compatible with AIH in biopsies of 92 of 1759 patients with chronic HCV infection [78]. However, other positions of the simplified IAIHG scoring system were not fulfilled: the median gammaglobulin level was not different from the other hepatitis C patients, and only a very small fraction of the 92 patients with interface hepatitis carried SMA or LKM antibodies. Although approximately 12% of all patients generated ANA there was no correlation with interface hepatitis, and application of the simplified IAIHG scoring system revealed only one single case of definite AIH [78]. Thus, rather than proving that HCV infection might lead to AIH, the study demonstrates that interface hepatitis is a histological pattern that is present in some patients with hepatitis C.

In the context of AIH-2, several independent studies demonstrated that LKM-1, the hallmark autoantibodies in AIH-2, are present in up to 10% of patients with a chronic HCV infection [23–25,79–81]. An interesting study has been performed with 60 LKM-1-positive and 120 LKM-1-negative patients with chronic hepatitis C [82]. LKM-1-positive patients had higher levels of gammaglobulin and a higher frequency of total intrahepatic CD8 T cells. Anti-viral therapy was equally effective in both groups. However, hepatic flares during therapy occurred rarely, but predominantly in LKM-1-positive patients [82]. These findings suggest that at least some of the HCV-infected patients might have also developed AIH. However, even if some of these patients indeed suffered from both hepatitis C as well as AIH, this observation is not proof that AIH has emerged as consequence of the HCV infection. In contrast to SMA and ANA the LKM-1 autoantibodies, which react to a more defined target autoantigen (CYP2D6), display a similar recognition pattern in patients with AIH-2 and chronic hepatitis C [76,83]. In addition, it has been found that some hepatitis C patients generated anti-HCV antibodies that are directed to the HCV proteins NS3 and NS5a and also cross-react to a conformational epitope on CYP2D6 spanning the amino acids (aa) 254–288 [84]. This region of CYP2D6 contains the immunodominant CYP2D6 epitope aa263–270 that has been found by us [85,86] and others [87–90] in AIH-2 patients and in the CYP2D6 model (see section below). Therefore, molecular mimicry between regions of HCV and CYP2D6 might be an explanation for the occurrence of such cross-reactive autoantibodies in chronic HCV-infected patients. An interesting case was reported after a child became infected with HCV after liver transplantation due to an α1-antitrypsin deficiency-related liver disease [91]. The patients generated LKM-1 antibodies within 2 weeks after transplantation, but no antibodies to HCV, although HCV RNA has been detected. As the patient did also not show histological evidence for hepatitis, the authors suggested that HCV infection might have triggered a cross-reactive primary immune response to LKM-1 target autoantigens but, because of the immunosuppressive regimen after transplantation, no AIH [91]. It is important to note that the mere presence of cross-reactive antibodies is not proof for HCV to be a triggering factor for AIH. There are also no solid data available that would document a higher frequency of HCV-specific antibodies in patients with AIH compared to the general population. In addition, it should be kept in mind that in the initial IAIHG scoring system from 1999, the presence of viral hepatitis was collated with a negative score [9].

An interesting aspect in the context of HCV infection and AIH might emerge from the recent success in the treatment of hepatitis C with interferon (IFN)-free direct-acting anti-viral (DAA) regimens [92]. It could be hypothesized that if there was a connection between HCV infection and AIH and molecular mimicry between HCV proteins and CYP2D6 this would indeed have an impact upon the immunopathogenesis of AIH, and that the full elimination of all HCV particles might influence the course of AIH (Fig. 2). In particular, the presence of HCV particles might keep cross-reactive antibodies and/or T cells busy with keeping the viral balance during a chronic HCV infection. It has been shown for several chronic viral infections that prolonged exposure to viral antigens causes T cell exhaustion characterized by reduced frequency and impaired effector function of CD8 T cells [93–95]. Thus, one could speculate that after HCV elimination such remaining cross-reactive T cells might regenerate and, over a long time-period, would slowly attack host structures with similarity to HCV (i.e. the mimicking epitopes on CYP2D6). Indeed, a recent case report states that an 81-year-old patient with chronic HCV infection developed AIH after DAA therapy [96]. The patient displayed elevated serum aminotransferase and gamma-immunoglobulin levels and the generation of ANA 2 months after beginning the DAA therapy. A subsequent liver biopsy revealed a typical interface and panlobular hepatitis with bridging fibrosis and plasmacytosis [96]. In contrast, another study demonstrated that DAA treatment of a 50-year-old patient with a chronic hepatitis C AIH overlap syndrome was successful in treating chronic hepatitis C without exacerbation of AIH [97]. However, the patient was first treated with prednisolone and only afterwards with DAA, resulting in a rapid decrease of both HCV-RNA as well as serum aminotransferase and gamma-immunoglobulin levels [97]. Thus, at present, the possibility for an underlying dormant AIH to be awakened by elimination of the deviating HCV

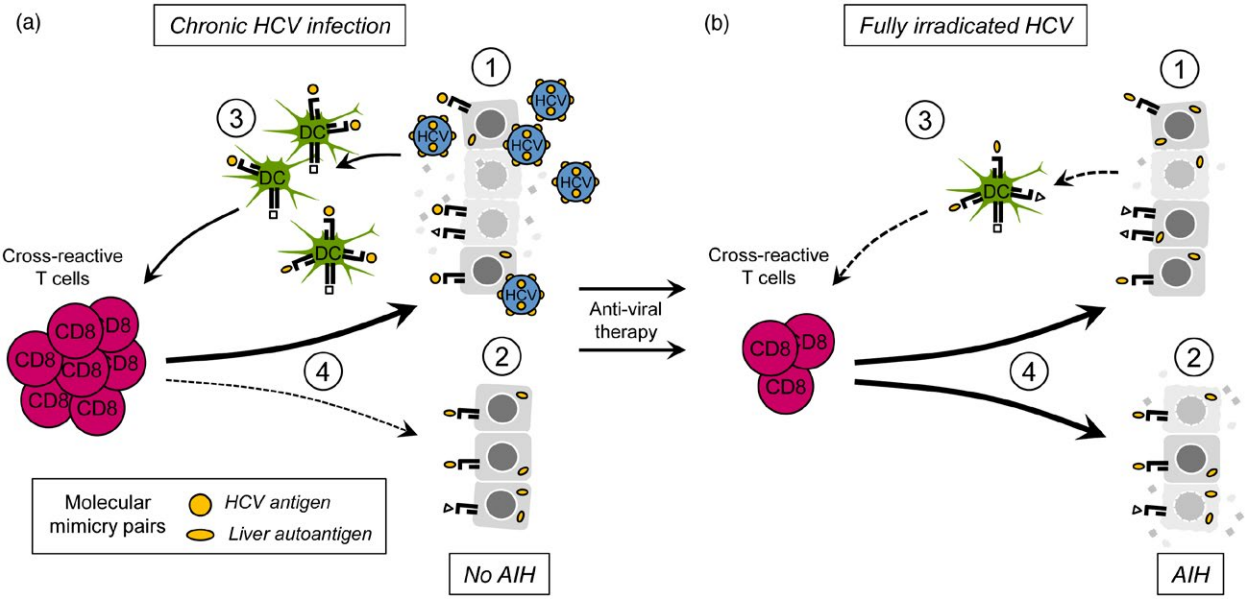


Fig. 2. Hypothesis: immune-deviation by hepatitis C virus (HCV). This figure describes a hypothesis of how molecular mimicry between HCV and a liver autoantigen might be involved in the aetiology of autoimmune hepatitis (AIH). Note that although an association between HCV infection and AIH has been suggested on many occasions, and liver/kidney microsomal (LKM)-1 autoantibodies have been found in a substantial fraction of hepatitis C patients, there is no proof that HCV might trigger AIH. (a) During chronic HCV infection the virus-specific immune response is in balance with the chronic viral load. Virus-infected hepatocytes present viral as well as self-proteins, including one that shares a structural similarity with a viral protein (1), whereas non-infected hepatocytes present endogenous antigens only (2). Professional antigen-presenting cells, such as invading dendritic cells (DC) and resident cells with antigen-presentation capacity, such as Kupffer cells, cross-present viral and self-antigens deriving from damaged hepatocytes (3). Cross-reacting T cells predominantly attack virus-infected hepatocytes (but rarely attack non-infected hepatocytes), resulting in chronic damage characteristic for HCV-infection, but not AIH. (b) Upon modern anti-viral therapy with direct-acting anti-viral (DAA) regimens HCV is eliminated completely, leaving behind only non-infected hepatocytes presenting self-proteins, including one that shares a structural similarity with a viral protein (1, 2). In this situation, antigen-presenting cells cross-present only endogenous antigens, resulting in a reduced activation of cross-reactive T cells. These T cells might have a lower avidity to the host protein that confers molecular mimicry than to the original viral antigen (3). Nevertheless, as the main target of the aggressive anti-viral immune response has disappeared, the remaining cross-reactive T cells now attack non-infected hepatocytes carrying the similar self-protein, resulting in AIH (4). Due to the presumably lower avidity of cross-reactive T cells to the self-epitope, this auto-destructive process may take a rather long time.

infection through DAA therapy remains purely an academic speculation. It will be interesting to follow-up on this thought when more long-term data from DAA-treated patients are available.

Epstein–Barr virus

Another interesting pathogen is Epstein–Barr virus (EBV), as it has been associated with the development of various autoimmune diseases, including systemic lupus erythematosus (SLE), multiple sclerosis (MS), autoimmune forms of thyroiditis (Grave's disease and Hashimoto's disease), rheumatoid arthritis (RA), inflammatory bowel disease (IBD), type 1 diabetes (T1D), Sjögren's syndrome (SjS) and myasthenia gravis (MG), as well as autoimmune liver diseases [98]. There are several case reports that describe the development of AIH following EBV infection; for example, a 26-month-old girl who, after a recent EBV infection, showed typical features of AIH, including elevated serum aminotransferase levels, the generation of LKM-1 antibodies as well as interface hepatitis with T cell/plasma cell infiltrations [99]. However, even though the EBV infection and the development of AIH were in close succession there was no proof for a causative relation of the two events. For EBV, in particular, proof is extremely difficult, as it is believed that up to 98% of individuals worldwide are infected with EBV and the virus remains persistent in most individuals [100]. An important prospective study was conducted by Vento *et al.* with a cohort of relatives of 13 AIH patients in whom two of seven women with a manifestation of EBV-induced infectious mononucleosis have also developed AIH-1 in a strict temporal relation [101]. The interesting feature about this finding is that both women already displayed an increased titre of LSP antibodies directed against the asialoglycoprotein receptor (ASGPR) before EBV infection. This increased titre was the result of a defect in a suppressor/regulatory T cell population controlling the immune

response to ASGPR [101]. Thus, EBV infection might have accelerated rather than initiated autoimmunity *de novo* in the liver, resulting in clinically overt AIH. Additional evidence for an association of EBV and AIH has been obtained from several other case studies [102–106]. In most of these cases the patients that presented initially with typical symptoms of acute EBV infections, such as severe sore throat, malaise, fever, lymphadenopathy, enlarged tonsils and pharyngitis, developed AIH shortly afterwards. There is one other case that is unusual with regard to the sequence of events: Chiba *et al*. found a chronic active EBV infection in a 22-year-old woman who had been diagnosed with AIH 6 years earlier [107]. The patient was treated with prednisone and cyclosporin A to treat AIH, but unfortunately died 10 months later due to EBV-associated haemophagocytosis and liver failure. A retrospective analysis of blood samples taken 6 years earlier had already revealed the presence of EBV genome and high-titre anti-EBV antibodies at the time of AIH diagnosis. [107]. Thus, EBV infection had occurred before AIH diagnosis and might have caused hepatic pathology similar to AIH [107]. In summary, evidence for a role of EBV in the aetiology of AIH is predominantly derived from case studies with single patients. Only very few studies have been conducted with larger cohorts of either newly EBV-infected individuals or with relatives of AIH patients. Due to the high frequency of EBV infections and the low frequency of AIH, a definite association is almost impossible to prove, but the documented case studies all have in common that the diagnosis of AIH was in close temporal proximity to EBV infection. In most cases the patients generated SMA and ANA, but rarely LKM-1 antibodies, indicating that EBV might be more likely to be involved in the aetiology of AIH-1 than of AIH-2. However, considering that AIH-1 is approximately 10 times more frequent than AIH-2, it might again be simply a question of AIH-subtype frequencies.

Other pathogens

Several other pathogens have been associated with AIH. One of the first associations was from two studies in the 1980s, when measles virus (MV) was found in 12 of 23 patients and 12 of 18 patients who had been diagnosed with AIH [108,109]. However, since the early 1980s the definition as well as the diagnostic tools for AIH have changed dramatically, and it is not clear if those patients would still be diagnosed with AIH today. In addition, as for EBV, the frequency of individuals who experience an infection with MV is extremely high. In fact, a subsequent study comparing AIH patients with the general population revealed no significant difference in the frequency of individuals who generated anti-MV antibodies [110]. It is interesting, however, that some case reports documented a strict temporal relation between MV infection and a subsequent emergence of AIH [111]. Thus, MV as well as EBV might be environmental factors triggering clinical AIH from a dormant autoimmune condition in a specific (molecular mimicry) or non-specific/bystander (general inflammation) manner.

Other associations have been reported for varizella zoster virus (VZV) infection [112] and cytomegalovirus (CMV) infection [113]. A 23-year-old man manifested with elevated serum aminotransferase levels, hypergammaglobulinaemia and generation of pANCA, as well as jaundice, 1 month after contracting VZV from a close family member. After initial successful treatment with prednisone for presumed AIH the serum aminotransferase levels increased again after corticosteroid withdrawal, and AIH was finally confirmed after analysis of a liver biopsy [112]. CMV infection was assumed to be a trigger of autoimmune liver disease in a 63-year-old woman with severe jaundice and elevated serum levels of bilirubin, aminotransferase, immunoglobulin and alkaline phosphatase [113]. As well as the presence of ANA and AMA, antibodies to CMV and EBV were detected [113]. Applying the revised scoring system of the IAIHG revealed no conclusive diagnosis, as the patient was carrying AMA and displayed chronic non-suppurative destructive cholangitis that both indicate PBC, rather than AIH. The authors suggested that CMV infection might have been a trigger for an AIH–PBC overlap syndrome [113]. One could speculate here whether the patient was already suffering from dormant PBC, and CMV infection in addition triggered AIH, resulting in an AIH–PBC overlap syndrome or vice versa.

Infection with human immunodeficiency virus (HIV) has also been suggested to trigger AIH [114]. Again, the evidence originates only from a case study in which a 52-year-old man with HIV infection displayed elevated serum aminotransferase and gamma-immunoglobulin levels, generation of ANA and interface hepatitis with plasmacytosis [114]. In contrast to most of the previously mentioned case studies, diagnosis of HIV infection and AIH were not in close temporal proximity, but were separated by 7 years. The patient was serum-negative for HAV-, HCV-, EBV- and CMV-related antibodies, but had also a medical history of a past recovery from HBV infection [114]. This case is interesting in so far as it might be an example for multiple virus infections that would be required for an autoimmune condition to result in an actual autoimmune disease. This ‘multiple hit’ theory has been suggested for other autoimmune diseases, such as type 1 diabetes or multiple sclerosis [61]. In addition, the impaired immune status of patients with HIV infection might be another factor that should be taken into consideration. The reduced number of CD4 T cells during

HIV infection as well as the treatment of chronically HIV-infected patients might have a selective impact on the regulatory T cell compartment and might thereby facilitate the development of an autoimmune condition. Indeed, it has been shown recently that treatment of HIV patients for several years with an intensified five-drug regimen containing maraviroc and raltegravir is associated with a reduced frequency of regulatory T cells [115].

Among non-viral pathogens, the parasitic *Leishmania* species have been associated with AIH in a recent case report [116]. A 26-year-old female patient presented with anorexia, malaise, weight loss and joint swelling as well as significant erythema and arthritis at ankles, wrists and hand joints. Due to the presence of elevated serum aminotransferase levels as well as ANA, SMA and AMA, together with an interface hepatitis with infiltrating plasma cells and lymphocytes, an AIH–PBC overlap syndrome was suggested and the patient was treated with prednisone and azathioprine [116]. After an initial recovery, a few weeks after the immunosuppressive regimen had been initiated her leucocyte and platelet counts dropped and neutropenic fever with persistent neutropenia and thrombocytopenia was diagnosed, requiring an antibiotic treatment. Finally, the patient was diagnosed with visceral leishmaniasis due to the presence of *Leishmania* amastigotes and parasites in bone marrow macrophages. Upon treatment with amphotericin B the fever resolved and blood counts returned to normal. Further, SMA were no longer detectable and only low titres of ANA and AMA were present. The authors suggested that the observed features of AIH and PBC might have been the result of the *Leishmania*-induced tissue destruction that might have caused a release of liver autoantigens and a subsequent generation of autoantibodies, including SMA, ANA and AMA [116]. However, the patient did not progress to develop a *bona fide* AIH, PBC or AIH–PBC overlap syndrome, and to date there is no firm proof that autoantibodies generated during the course of AIH are directly pathogenic [13]. Interestingly, in the context of parasite infections there has been no association reported between AIH and helminth infection. This circumstance would be in line with the current opinion that helminths, which predominantly elicit a type 2 T cell response, might protect from, rather than induce/accelerate, autoimmune diseases [60].

In summary, firm proof for pathogens to act as environmental factors triggering AIH is still missing. Most of the evidence is of an anecdotal nature, originating from several case studies describing observations in one single patient with AIH who had previously experienced an infection with pathogens, such as EBV or HCV. Although cross-reactive antibodies reacting to both viral as well as host antigens have been found in some patients, there is still no proof that the associated pathogens are indeed capable of inducing AIH *de novo* or alternatively to accelerate a pre-existing, dormant autoimmune condition.

Searching for a trigger for AIH

Many associations between pathogen infection and AIH have been made based on single case studies with patients who have developed AIH in close temporal proximity to a pathogen infection. However, when considering that the time elapsed between infection and precipitation of the disease might be years or even decades, finding an association is much more difficult, in particular if exposure to more than one pathogen might be required to elicit the disease. Therefore, one possibility might be that an infection with a pathogen, which may or may not confer molecular mimicry to host autoantigens, might have occurred long before diagnosis, but had initiated an autoimmune reactivity resulting in a dormant form of AIH. Later in time, a second infection with the same or another pathogen would then precipitate the disease, which would be clinically apparent shortly afterwards. Whereas the second infection would be easy to track, traces of the first infection might have been long gone by the time of diagnosis. Thus, another approach of finding pathogens that might be involved in the aetiology of AIH is to analyse the immune response to the liver autoantigens in more detail. If molecular mimicry between pathogen and host plays a role, cross-reactive antibodies and/or T cells should be present in patients with AIH. Knowing the precise epitopes that are recognized by these cross-reactive antibodies would then allow for screening of pathogens that share a similar epitope.

CYP2D6 is the major target autoantigen recognized by LKM-1 antibodies in AIH-2 patients, and the immune response to CYP2D6 has been investigated in great detail during the last decades. Many epitopes have been reported to be recognized by different fractions of patients. The immunodominant CYP2D6 B cell epitope was mapped by several groups in the early 1990s, and its dominance in terms of frequency among patients as well as magnitude of the generated titres has been confirmed in many studies [85–90]. The immunodominant epitope spans a region of high antigenicity located at amino acids 254–271 (aa254–271). Antibodies specific for this entire region, or parts of it, have been detected in the majority of patients, ranging from 62 to 100%, depending on the individual study [85–90]. However, several other CYP2D6 epitopes have been detected, and might play a role in the aetiology of the disease. These epitopes include regions aa321–351, aa373–389 and aa410–429 [90]; aa196–218 [117]; aa193–212, aa238–257, aa268–287 and aa478–497 [118];

aa55–63, aa139–147, aa203–211, aa239–aa247 and aa379–aa429 [86], aa284–391, aa412–429 and the conformational epitopes aa1–146 [119] and aa321–379 [120]. It has to be kept in mind that the reason for a dominance of sequential epitopes lays in the methods that have been used for identification. Most of such methods use linear peptides in membrane-bound or soluble form, and therefore conformational epitopes are rarely detected. Whereas T cell epitopes are, due to the mechanism of antigen presentation within the MHC binding grooves, predominantly linear, B cell/antibody epitopes are mainly dependent upon the surface accessibility on the antigen, and may also contain a considerable portion of conformational epitopes.

Many of the listed epitopes share a sequence homology to one or more human pathogens [86,87,121]. The immunodominant epitope aa254–271 recognized by the majority of AIH-2 patients contains a core sequence PAQPPR that is also present in the infected cell protein 4 (ICP4) of human alpha herpesvirus 1 (HHV-1) [89], indicating that HHV-1 infection might play a role in the pathogenesis of AIH. Bogdanos *et al*. demonstrated that ICP4 of HHV-1 was also recognized by sera from chronic hepatitis C patients with LKM-1 antibodies [121]. Surprisingly, however, the serum reactivity to ICP4 was independent of the LKM-1 status, and antibody inhibition studies revealed true cross-reactivity in only two of 23 LKM-1 antibody-positive hepatitis C patients [121]. Another prominent sequence homology has been reported for the epitope aa193–212, that shares homology with the RNA-dependent DNA polymerase NS5 sequence aa2977–2996 of HCV, and the alkaline exonuclease sequence aa121–140 of CMV [118]. In contrast to HHV-1, an association between AIH and HCV and CMV has been reported (see above section).

One problem for the identification of epitopes that might play a role in the aetiology of AIH is the time of serum retrieval. At the time of diagnosis, when for most patients the first blood sample is taken and stored, the serum reactivity to the first immunogenic epitopes that had been recognized at initiation of the antigen-specific immune response might have disappeared. A mechanism termed 'epitope spreading' is held responsible for such a phenomenon, in which the immune reactivity is spreading from an initial epitope to neighbouring or even remote regions of the antigen. In the context of an infection with a pathogen conferring molecular mimicry to a host autoantigen, the mimicking epitope might be responsible for the initiation of the immune reactivity, but it might not necessarily be the immunodominant epitope recognized at the time of diagnosis. Thus, an analysis of patients' sera at or after diagnosis might reveal only the immunodominant, but not the initiating, epitopes. As it is extremely difficult to acquire stored serum samples collected before diagnosis, we used an animal model for AIH that displays a very similar B cell/antibody immune response to CYP2D6 as do patients with AIH-2. In the CYP2D6 mouse model, wild-type mice (FVB or C57BL/6) are infected with an adenovirus encoding the human CYP2D6 (Ad-2D6) [85,122]. As wild-type mice do not express human CYP2D6, but mouse Cyp homologues (Cyp2D8, Cyp2D11, Cyp2D22 and Cyp2D26), the Ad-2D6-mediated expression of human CYP2D6 breaks tolerance to the similar, but not identical, mouse Cyp homologues (molecular mimicry). The Ad-2D6-infected mice develop persistent AIH-like disease characterized by cellular infiltrations in the peri-portal area, fibrosis and the generation of CYP2D6-specific autoantibodies, as well as specific T cells that accumulate in the liver [85,122–124]. In contrast to many other AIH models [125], the main advantages of the CYP2D6 model are the use of an actual human autoantigen and its inducibility. With regard to investigating initiating epitopes and determinant spreading, the precise starting point of the anti-CYP2D6 immune response is known. Mapping the entire CYP2D6 sequence for B cell/antibody epitopes at several times after infection of mice with Ad-2D6 revealed that the immunodominant epitope spanning aa259–270 (RMTWDPAQPPRD) found for LKM-1 antibodies generated by AIH-2 patients was also the first epitope to which the mice generated antibodies [86]. Just as detected for AIH-2 patients from whom serum samples have been collected during a large period of time (more than 10 years after diagnosis), the immunodominant aa258–270 epitope remained dominant throughout the observation time. In addition, several other epitopes were recognized over time and a well-pronounced determinant spreading was detected that covered neighbouring as well as remote locations of the CYP2D6 molecule [86]. Like the immunodominant aa258-270 epitope, most of those subdominant epitopes are also located at the surface of the CYP2D6 molecule. Interestingly, five of the six epitope regions that have been recognized in sera of AIH-2 patients have also been recognized by Ad-2D6-infected mice at the end of the observation time [86]. Screening of the NCBI GenBank revealed sequence homologies to many different human pathogens, including some that have been already associated with AIH, such as HCV, HIV and CMV. In addition, sequence homologies to pathogens that have not yet been connected to AIH have been identified, including rabies virus, Karposi's sacrcoma-associated herpes virus (HHV-8), *Legionella pneumophila* and several *Mycobacterium*, *Burkholdria* and *Brucella* species [86]. Thus, sequence similarities between CYP2D6 epitopes and pathogens are frequent and are not restricted to HHV-1 and HCV.

In addition to CYP2D6-specific antibodies, autoreactive CD4 and CD8 T cells specific for CYP2D6 were found in

the blood and liver of AIH-2 patients [126,127]. A detailed T cell epitope mapping of CYP2D6 revealed CD4 T cell reactivity to seven antigenic regions in *DRB1*07:01* and four regions in non-*DRB1*07:01* patients [44]. Among these T cell epitopes, the region aa313–332 shares a sequence homologue to polyprotein 1A aa794–801 of HCV [128]. A similar study has been conducted for CD8 T cell epitopes in HLA-A2-positive and -negative patients with AIH-2. After algorithm prediction of HLA-A2-binding CYP2D6 peptides, five different HLA-A2-CYP2D6 peptide tetramers have been generated and used to detect CYP2D6-specific CD8 T cells in patients [129]. The dominant CD8 epitopes turned out to be located in the region aa245–254, which flanks the region of the immunodominant B cell/antibody epitope aa254–271 and overlaps with a previously identified CD4 T cell epitope aa225–260 [44]. The CYP2D6 region qq245–254 shares a sequence homology with uroporphyrinogen-III C-methyltransferase, an enzyme that is required for cysteine synthesis in enterobacteria, such as several *Escherichia coli* as well as *Hafnia* and *Erwinia* species. However, been no association has been reported that would link these enterobacteria to AIH.

Conclusion

Several case studies have associated pathogen infection with the development of AIH. Often, such infections were in close temporal proximity to the diagnosis of AIH. However, a causal relationship between pathogen infection and AIH has yet to be proved. Proof, however, is difficult, as some pathogens are widespread in the population, and remaining traces of the pathogen, such as viral RNA and/or pathogen-specific antibodies, are found in patients with AIH as well as in healthy individuals. Further, the general belief that a certain genetic predisposition and/or multiple pathogen infections might be required to induce and/or accelerate an autoimmune condition further complicates the search for evidence. Another factor that has to be taken into account is the reporting bias that might have an influence on some of the statistical data available. In particular, the strong desire of both patients and treating physicians to identify a causal relationship in a disease whose pathogenesis is not fully understood might lead to certain misrepresentations of two merely coincidental events.

Evidence obtained from animal models, including the CYP2D6 model as well as others, suggests that an infection with a liver-trophic pathogen is most probably required to induce an AIH-like state. Animal models that use transgenic model autoantigens and presence of TCR-transgenic T cells mainly require an additional infection with liver-trophic pathogens, such as lymphocytic choriomeningitis virus (LCMV) [130] or *Listeria monocytogenes* [131]. In the CYP2D6 model, the generation of CYP2D6-specific B cells/antibodies and T cells can also be achieved by subcutaneous injection of recombinant human CYP2D6 and complete Freund's adjuvant. However, the mere generation or presence of such a reactivity to CYP2D6 was not sufficient to cause liver damage and AIH-like disease, indicating the requirement to elicit a local inflammation in the liver by a liver-trophic pathogen [124].

Viruses cause a robust type 1 T cell response that targets virus-infected cells with the goal of eradicating the pathogen. Thus, it is no surprise that viruses such as HAV, HCV and EBV are the pathogens that have been extensively discussed to be involved in the immunopathogenesis of AIH [98,132]. Molecular mimicry between a liver trophic virus and liver autoantigens is a feasible hypothesis that would explain the breakdown of liver tolerance. The molecular mimicry concept has been evaluated in many models of autoimmune diseases [64], including AIH [85,122], demonstrating an initiation and/or acceleration of the autodestructive process. However, with a few well-documented exceptions [69,70], no firm proof has been delivered. It is therefore of upmost importance that large, multi-national prospective studies are conducted that aim at finding environmental factors, including pathogen infections, diet, vaccination, exposure to drugs, pollution, stress and many other factors that might have an association with AIH. Such studies are currently ongoing for other autoimmune diseases, such as 'The Environmental Determinants of Diabetes in the Young' (TEDDY) study (website: teddy.epi.usf.edu) for type 1 diabetes. Only with data from such large cohorts of patients will it be possible to, on one hand, identify factors that play a role in the aetiology of AIH, and on the other hand find targets for immune intervention in patients.

Disclosures

The authors confirm that they have no competing interests.

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